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Handling Factors Not Body Marking Influence Thalamic Mast Cell Numbers in the Preweaned Albino Rat

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In order to determine whether body marking or the stimulation associated with this procedure alters mast cell numbers in the rat brain, a total of 60 rats (*Rattus norvegicus*) were selected from both split litter and litter designs (total of 15 litters). Subjects from the split litter design had been either: (1) marked daily starting at 5 days of age with either a permanent ("toxic") marker or water color ("non-toxic") marker over the dorsal rear body surface, or (2) not marked but stroked with the experimenter's thumb over the area for a similar period of time. In the litter design, entire litters were either stroked and handled daily (like the split litter controls) or not handled. No significant difference in mean numbers of thalamic parenchymal, leptomeningeal or total mast cells in either 15- or 20-day-old brains were noted between rats that had been marked with the two materials or between marked or nonmarked (but stroked) rats. However, rats that had been handled daily in the litter design displayed less than one-half the numbers of brain mast cells found in rats that were not touched daily ($p < .001$). Handling alone could account for about 50% of the variance in mast cell numbers within the thalamic nuclei. Highly significant differences in numbers of mast cells present within the leptomeninges (or parenchyma) were noted between the 15- and 20-day-old brains.

Thousands of mast cells (MCs) have been reported within the diencephalic portions of the young rat brain (Dropp, 1974; Krueger, 1974; Persinger, 1977a). These clear, metachromatically staining cells display ontogenetic alterations in numerical distribution. Whereas the 15-day-old rat brain contains most MCs within the diencephalic leptomeninges and processes of the subfornical organ, older brains demonstrate a progressive increase in MCs within thalamic parenchyma, particularly within the ventral and anteroventral nuclei (nomenclature after Konig and Klippel, 1963).

Since (1) the number of these cells in the brain could account for total juvenile brain histamine levels (Dropp, 1976; Schwartz, 1975), (2) these

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cells appear to concentrate around blood vessels, and (3) nonneural MCs are involved with neuroimmunological processes in many species, the possibility that the numbers of these cells in the parenchyma could be modified by early experience (behavioral stimuli) was considered important.

Although Persinger (1977b) reported that preweaned rats that had been body marked as a part of various treatment procedures displayed fewer brain MCs than nonmarked littermates, there are alternative data interpretations. Was the MC reduction associated with the potentially toxic materials in the marker, as suggested by Padawer (1975)? Since the control (nonmarked) pups in that study were handled briefly and not held firmly and stroked on the rear dorsal surface (associated with application of the marker), were stroking and/or protracted constraint confounding factors? To answer these questions and to determine the reliability of original report, this experiment was completed.

METHOD

Subjects

Thirty 15-day-old and thirty 20-day-old albino Wistar rat pups were used as subjects. Their parents had been 60 days of age when they were obtained from Bio Breeding Laboratories, Ottawa, Ontario, Canada.

Procedure

A total of 15 pregnant 110- to 130-day-old (primiparous) females from four separate one-night breeding sessions separated by one week were removed from standard 41 × 24 × 18-cm wire cages (three/cage) about 3–4 days before predicted birth and placed individually in 41 × 25 × 18-cm plastic cages containing 15-cc of 0.6 cm corncob bedding. Within 24 hr after parturition, all litters were culled to eight pups. On postnatal Day 5, the litters were allocated to one of four groups.

The first two groups (eight litters) involved a split litter design. Daily, between postnatal Days 5 and 20, mothers were removed from their litters and housed separately for 15 min. Each pup was then removed singly, placed on the lid (covered with one layer of tissue to prevent excessive heat conduction) for 20 sec, weighed, and then treated with the experimental or control procedure for 40 sec. Treatments were applied over the dorsal surface of the rear half of the pup (primarily over the spinal cord region) while it was held firmly within the experimenter's left hand.

Half the numbers of pups (equalized for sex) in four of the litters received daily applications of a permanent Sanford deluxe blue magic marker No. 1000 that contained xylene–toluene and related substances (see Persinger, 1977b). Half the numbers of the pups in the other four litters were treated with a nonpermanent Mr. Sketch No. 2000 blue marker containing a water color base. Marked areas were 2.3 to 3 cm^2.

Whereas the permanent marker produced drying and scaling of the skin (although hair growth differences were not apparent), the water color marker produced no obvious skin alterations. The second pair of litters from each marking type were marked by a second appropriate marker in order to attenuate possible effects from a single marker.

The remaining pups from these litters served as marking controls. Unlike the previous study (Persinger, 1977b), these pups were weighed for 20 sec and then restrained and rubbed with the experimenter's thumb on the rear dorsal surface (simulating the marker movement as much as possible) for 40 sec. Since the toxic marker required only four applications while the watercolor-based marker required eight applications to maintain the color for 24 hr, the controls were given the appropriate number of thumb strokes.

On alternate days, all of the marked pups in each litter were treated first while on other days the nonmarked (stroked pups) were treated first. Following treatment, each pup was placed in a small box (10 cm all sides) containing bedocob. After postnatal Day 15, they were placed in a plastic cage for that period. When all pups for a litter had been treated, the litter and the mother were returned to the main cage. Elapsed time for separation from mother was between 10 and 15 min daily.

The second group of seven litters was allocated to a litter design. Each pup from four of the litters (selected from the four breeding sessions) was given the control treatment: handled and stroked over the dorsal rear surface by the experimenter's thumb for 40 sec following 20 sec on the pan balance. The remaining procedures were the same as the animals in the split litter designs.

However, the other three litters were never handled, except when the bedding was changed on Days 10, 15, and 17. During this time, the whole litter was removed from the mother simultaneously for about 2 min. The pups were picked up quickly and handled not more than 5 sec during the entire change. Such procedures had been used previously (Persinger, 1979).

All treatments were given between local time 1200 and 1600 hr. Bedding was changed in the handled litters every second day between postnatal Day 10 and 20 immediately after the treatment period so that pups and mothers were returned to fresh bedding. Fresh water was given daily and Purina rat food available ad libitum.

The following colony conditions existed throughout the experiment: L:D cycle, 12:12 (onset 0800 local time); temperature 22 ± 1°C and relative humidity 45 ± 5%. All litters were maintained in the same room that also contained breeder males. Except for the first four litters, all consequent pregnant females and litters were exposed to the smells and other stimuli associated with lactating females and rat pups of different ages.

On postnatal Days 15 and 20 (10 and 15 days of treatment, respectively), a marked and nonmarked rat from each litter in the split litter design (one male and one female; the sex of the marked or nonmarked animal selected on Day 15 or 20 was alternated for each consecutive litter of that treatment) and two pups from each of the daily, handled-stroked and nonhandled litters in the litter design were decapitated within 5 min of initial nest disturbance on that day. The time since the last treatments was 24 hr, in order to attenuate possible acute effects.

The brains of the rats were removed quickly (2 min) with minimal mechanical deformation and the cerebrums were fixed in either formalin (10% formaldehyde; 10% aqueous CaCl) or EFA (90 parts 80% ethanol; 5 parts 30% formalin, and 5 parts glacial acetic acid); fixatives were counterbalanced for sex, treatment, and age. After dehydration (2 × 2 hr 95% ethanol, 2 hr 100% ethanol, 12 hr 100% ethanol, and 2 hr 100% ethanol), clearing (2 × 1.5 hr chloroform), and paraffin embedding (3 × 2 hr), the tissues were sectioned (coronally) at 10 μm.

Nine sections, equally spaced between the posterior level of the posterior medical geniculate and the anterior ventral nuclei of the thalamus/subfornical organ, were selected for each rat and strained for mast cells, as defined by both metachromasia and morphology at 400×, by toluidine blue O (Humason, 1972). The EFA fixation–staining procedure allows clear differentiation of mast cells (purplish to pinkish red) against a pale blue background; the formalin fixative tends to produce a much darker blue background and more difficult discrimination as well as fine tissue fragmentation.

The entire diencephalic surface area of each section was traversed at 400× using an Olympus microscope (trinocular Model EC). Total numbers of MCs, recorded by a handcounter for each 10-μm section, was determined within the leptomeninges (Fig. 1) and the diencephalic parenchyma (Figs. 2 and 3). Other procedures used to insure accurate counting have been reported (Persinger, 1979). Sections from all the rats of the split litter design and from half of the rats of each condition in the litter design was counted by this experimenter; recount variability was less than 2%. As an extra control, a technician counted the remaining slides from the litter design.

The means for the leptomengeal, parenchymal, and total MCs for each brain were calculated by averaging the values from its nine slides. As an extra check for internal reliability, separate means were calculated for the three triad of slides that included the levels of the ventral nuclei, habenula, and anterior nuclei of the thalamus.

Four-way analyses of variance (ANOVA) for mean: leptomeningeal, parenchymal, and total (leptomeningeal plus parenchymal) MCs were completed as a function of marking condition (marked vs nonmarked but stroked), marker type (permanent vs nonpermanent color), age (15- vs

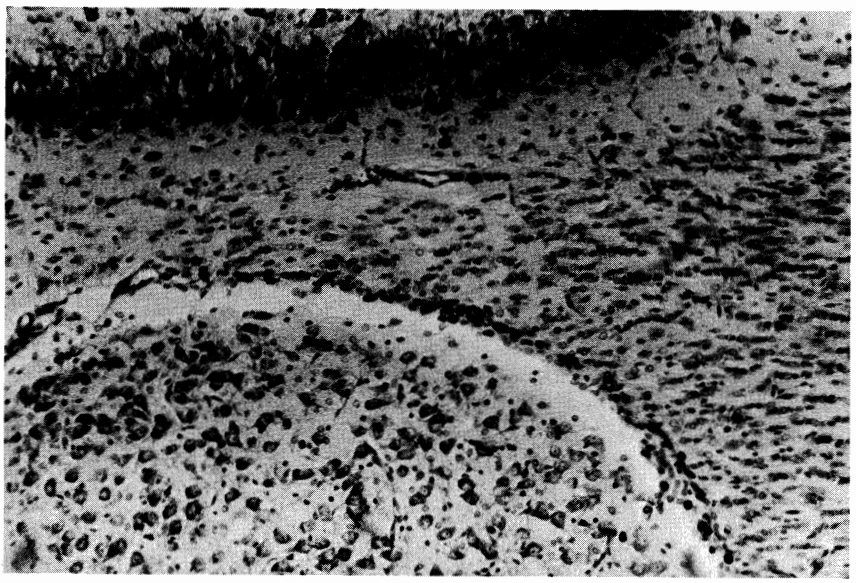

FIG. 1. Mast cells (dark elements) distributed within the diencephalic leptomeninges at the interface of the fimbria and the anterior thalamus in a 15-day-old rat (100×, toluidine blue O stain).

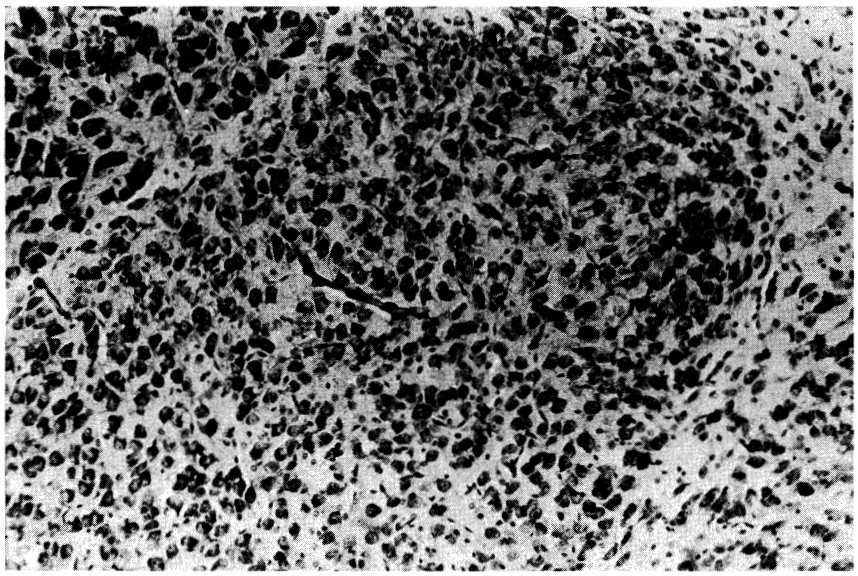

FIG. 2. Mast cells (dark elements) distributed along the blood vessels of the anteroventral nucleus of the anterior thalamus in a 20-day-old rat (100×, toluidine blue O stain).

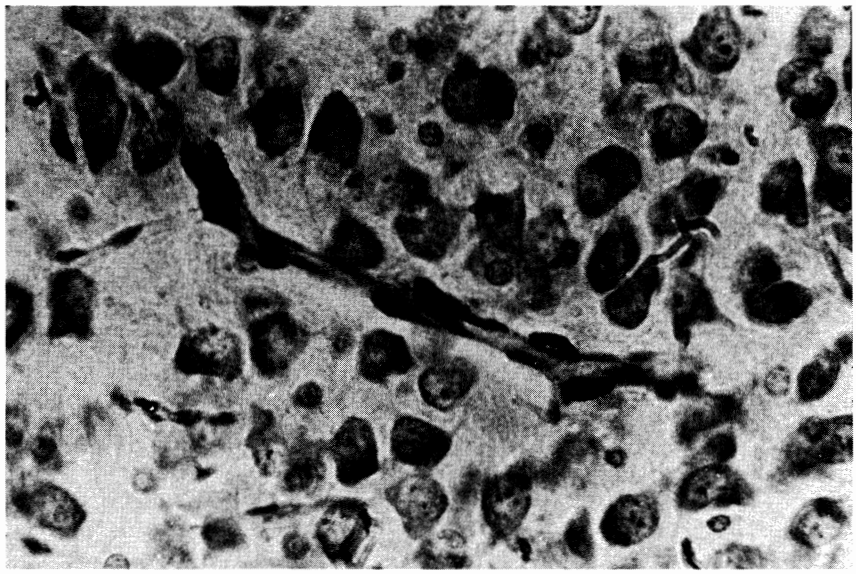

FIG. 3. Magnified (400×) display of nine mast cells along a blood vessel in the anteroventral nucleus of the anterior thalamus. Note evident granular characteristic of the MCs even at this magnification (toluidine blue O stain). The larger, irregular-shaped parenchymal elements are the cell bodies of neurons while the smaller more circular elements are nuclei of glial cells.

20-day-old), and sex (male vs female). For the litter design, three-way ANOVAs were conducted on these measures for handling (handled-stroked vs no daily handling), age (15- vs 20-day-old) and sex (male vs female).

All analyses, including ad hoc one-way ANOVAs, means, standard deviations, and correlation coefficients were computed by Dec 2020 computer using SPSS programs. Data check programs were completed to detect possible data loading errors. Sample means and one-way ANOVAs for main effects were checked manually.

RESULTS

Means and standard deviations for the mean numbers of mast cells per 10-μm section in the diencephalic:leptomeninges, parenchyma, and total area[2] for the pups from the split litter design are shown in Table 1. Four-way analyses of variance (for marking, marker type, sex, and age), demonstrated only one significant main effect. The 15-day-old pups (n = 16) displayed significantly more leptomeningeal MCs than the 20-day-old (n = 16) pups ($F(1, 16) = 26.54, p < .001$). However, the 20-day-old pups

[2] No MCs were noted in any of the hypothalamic nuclei within the boundaries studied.

TABLE 1
Means and Standard Deviations for Body Weights and for Mean Numbers of MCs per 10-μm Section in the Leptomeninges around the Diencephalon, the Diencephalic Parenchyma, and Combined Measures for Rats in the Split Litter Design according to Marking, Marker Type, Age, and Sex Conditions

		Marked		Marker		Age (days)		Sex	
		No	Yes	W	P	15	20	M	F
MCs	*N:*	16	16	16	16	16	16	16	16
Leptomeninges									
$\bar{X}$		31.1	29.8	30.9	30.1	43.9[a]	17.0[c]	31.9	28.9
SD		20.8	16.2	18.9	18.3	16.1	7.2	19.4	17.7
Parenchyma									
$\bar{X}$		19.7	18.9	21.1	17.5	11.3[a]	27.3[b]	21.3	17.3
SD		14.9	21.3	21.6	14.2	12.1	19.8	19.4	17.1
Total									
$\bar{X}$		48.6	48.8	52.0	46.4	54.0	44.3	53.3	45.0
SD		27.3	25.2	27.8	24.3	26.1	25.4	26.2	25.5
Body weight									
$\bar{X}$		42.6	42.8	42.4	43.0	35.5[a]	49.9[c]	44.0[a]	41.4[b]
SD		7.8	8.1	7.9	8.3	1.4	4.0	9.5	5.8

Note. W, watercolor; P, permanent; for both marked and non-marked pups. *a* vs *b* = $p \leq .01$; *a* vs *c* = $p < .001$.

showed significantly more MCs in the parenchyma than the 15-day-old pups ($F(1, 16) = 6.12, p = .02$). The number of total number of MCs in the diencephalon did not differ significantly. None of the interactions were statistically significant.

Means and standard deviations for the mean numbers of MCs per 10-μm section in the diencephalic:leptomeninges, parenchyma, and total area for the pups from the litter design are shown in Table 2. Three-way ANOVAs (for handling, age, and sex) demonstrated statistically significant handling and age differences. Handled-stroked pups displayed fewer MCs in the leptomeninges ($F(1, 20) = 8.18, p < .01$), in the parenchyma ($F(1, 20) = 26.70, p < .001$), and total MCs ($F(1,20) = 16.74, p < .001$) than nonhandled rats. Fifteen-day-old rats showed more leptomengeal MCs ($F(1,20) = 27.70, p < .001$) and total MCs ($F = 1,20) = 8.10, p < .01$) than the 20-day-old pup brains. The interactions were not statistically significant.

The amount of total variance in MC numbers that can be accounted for by total design (four- or three-way ANOVAs) are shown in Table 3. Ω^2 estimates (after Hays, 1963), of "explained" variance from one-way ANOVAs performed as a function of age only or handling only are also displayed in Table 3. Age as a single variable was found to account for most of the explained variance in leptomeningeal MC numbers but not in parenchymal MC numbers.

TABLE 2
Means and Standard Deviations for Body Weights and for Mean Numbers of MCs per 10-μm Section in the Leptomeninges around the Diencephalon, the Diencephalic Parenchyma, and Combined Measures for Rats in the Litter Design according to Daily Handling, Age, and Sex Conditions

		Handled and stroked		Age		Sex	
		Yes	No	15	20	M	F
MCs	*N:*	16	12	14	14	14	14
Leptomeninges							
$\bar{X}$		25.5[a]	41.3[b]	47.3[a]	17.2[c]	26.9	37.6
SD		16.7	25.1	21.1	7.1	17.5	24.9
Parenchyma							
$\bar{X}$		8.0[a]	24.9[c]	12.3	18.7	13.2	17.2
SD		6.8	11.1	11.7	12.3	12.1	12.3
Total							
$\bar{X}$		33.5[a]	66.2[c]	59.7[a]	35.3[b]	40.1	54.9
SD		17.7	29.3	32.1	17.4	23.1	31.6
Body Weight							
$\bar{X}$		39.9	41.8	33.8[a]	47.6[c]	42.2	39.2
SD		9.1	7.2	3.8	4.8	8.3	8.1

Note. *a* vs *b* = $p \leq .01$; *a* vs *c* = $p < .001$.

Four- or three-way ANOVAs performed at each of the three levels of the diencephalon, as an internal check for possible extreme value distortions, demonstrated that all nonhandled rats in the litter design had three to four times more MCs in the parenchyma at all levels than the handled rats. The pattern for 15-day-old rats to have twice as many MCs in the leptomeninges than in the parenchyma and for the 20-day-old rats to have twice as many MCs in the parenchyma than in the leptomeninges was evident across all three levels for subjects in both the split litter and litter designs.

The means and standard deviations for the body weights on the kill day as a function of the different main conditions for the split litter and litter designs are presented in Tables 1 and 2, respectively. Highly significant age differences were found for body weight ($F(1,16) = 239.14$, $p < .001$) and ($F(1,20) = 56.95$, $p < .001$, respectively). Body weight differences were significantly different between the sexes ($F(1,16) = 7.45$, $p < .01$) in the split litter design but not in the litter design. The only significant interaction (sex by age) was found in the split design ($F(1,16) = 13,87$ $p < .001$, and was a consequence of the male rats heavier weight gains than female rats as a function of age.

Correlations (r) between body weight and leptomeningeal, parenchymal, and total MCs for all 60 pups were: $r = -0.67$ ($p < .001$), $r = 0.37$ ($p < .01$), and $r = -0.27$ ($p = .01$), respectively. However, analyses of the

TABLE 3
Total Explained Variance according to the Major ANOVAs (four three-way) and according to Single, One-way ANOVAs (as comparison) for MC Numbers in the Diencephalic Leptomeninges, Parenchyma, and Combined Measures for the Split and Whole Litter Designs

	Split Litter		Litter		
MCs	Total explained (four-way ANOVA) (%)	Age (one-way ANOVA) (%)	Total explained (three-way ANOVA) (%)	Age (one-way) (%)	Handling (one-way) (%)
Leptomeninges	66	55	67	49	10
Parenchyma	46	20	63	6	46
Total	30	4	60	15	31

scattergrams demonstrated that these correlations reflected the two distinct numerical populations of body weights associated with 15 and 20 days of age. When correlations were computed between the MC measures and body weights for the 15-day (n = 30) and 20-day (n = 30)-old pups separately, no significant coefficients ($r < \pm 0.20$) were obtained.

One-way ANOVAs for mean leptomeningeal, parenchymal, and total MCs as a function of: birth sequence (n = 4), marking series (n =2), mean weight of the litter on postnatal Day 5, number of pups in the litter before culling (10 to 14), fixative or cytometrician (for the litter designs), differences were not significant statistically. Correlation coefficients (r) between leptomeningeal and parenchymal MCs for the 15-day (n = 30) and 20-day (n = 30)-old pups were +0.75 and +0.62, respectively.

Separate analyses for litter differences indicated that the litter with the fewest mean numbers of total MCs differed by a factor of 2 to 4 from those with the highest MC numbers. Whereas the mean numbers of total MCs for the eight litters (n = 4/litter) in the split litter design averaged 49.2 ± 25.8, the mean numbers of total MCs per 10 μm for the litters in the litter design averaged 47.5 ± 28.2. The litters with the lowest and highest MC numbers for the split litter and litter designs were: 21 ± 4 and 30 ± 18 and 82 ± 39 and 65 ± 32, respectively. Ω^2 estimates indicated that only 20% of the variance in the split litter design could be explained by litter differences while 35% of the variance in the litter design (but confounded by the handling treatment) could be explained by litter differences.

DISCUSSION

The characteristics of MC distributions and absolute numerical values are compatible with several other studies. The means and standard deviations for MCs per 10 μm section for the 32 brains in the split litter design and the 28 brains in the litter design were 49 ± 26 and 48 ± 29, respectively. Means and standard deviations for MCs per 10 μm (No. of subjects in parentheses) in other studies have been: 36 ± 16 (n = 16), 56 ± 40 (n = 36), and 32 ± 19 (n = 34) (Persinger, 1977b, 1979, 1980, respectively). As before (Persinger, 1979), (1) litter differences accounted for about 25% of the variability in MC numbers and (2) there were no sex differences in MC values.

Clear changes in MC numbers within the diencephalic leptomeninges and thalamic tissue as a function of age were quite evident; in fact, age alone accounted for about 50% of the variability in MC numbers within the leptomeninges (Ω^2 estimates). The mean percentage and standard deviations of total MCs in the parenchyma (No. of subjects in parentheses) for the split litter and litter designs were 18 ± 11% (16) and 18 ± 8% (14) for the 15-day-old pups and 57 ± 12% (16) and 46 ± 15% (14) for the 20-day-old pups, respectively. In the 1977b study, the percentages of total diencephalic MCs in the parenchyma were 18% (8) and 54% (8) for

the 15- and 20-day-old pups. Thirty-four 21-day-old rat brains from another study (Persinger, 1980) demonstrated 55 ± 15% of the total MCs in the thalamic parenchyma.

These data suggest that the brain MC in the rat may migrate from the leptomeninges to the parenchyma especially between postnatal Days 15 and 20. One is impressed by the apparent reversal of MC ratios in the thalamic parenchyma and leptomeninges as a function of age. For example, the ratios of MC numbers in the 15-day-old brain to MC numbers in the 20-day-old brains of nonmarked, marked, handled, and nonhandled and all 1977 rats for the leptomeninges were 3.1, 2.1, 2.9, 2.7, and 2.4; for the parenchyma, the ratios were 0.7, 0.2, 0.6, 0.7, and 0.5.

At a neurobiological level, there are several unanswered questions about the appearance of these presumably histamine-heparin containing cells in the rat brain. It is not clear whether the MCs that disappear from the leptomeninges are the same MCs that appear in the parenchyma. Since the 15-day-old brain contains about 25 to 50% more total MCs than the 20-day-old brain, what happens to the remaining MCs?

The possibility that behavioral manipulations during preweaning development could evoke significant alterations in MC numbers (and perhaps distribution) is supported by several independent data sources. In the 1977 study, the eight marked rats averaged 24 ± 8 MCs per 10 μm while the nonmarked (and less handled) rats averaged 47 ± 14 MCs per 10 μm. The 16 handled rats in the present study from four different litters averaged 34 ± 18 MCs per 10 μm while the 12 nonhandled rats from the three different litters averaged 66 ± 29 MCs per 10-μm section.

An independent (but ad hoc) verification that behavioral manipulations during preweaning development can produce a one-half reduction in MC numbers is evident. A comparison of the 36 rats (five litters) from Persinger (1979) and 34 rats (five litters), from another study (Persinger *et al.*, 1980) demonstrated mean MCs per 10 μm of 56 ± 40 and 32 ± 19, respectively ($t(1,68) = 3.16$, $p < .005$). The major differences between these two experiments involved early handling procedures. Whereas the rats in the first (1979) study were never handled (except for two manditory bedding changes), the litters in the second (1980) study were disturbed, weighed, and restrained (for injections, e.g.) daily between postnatal Days 14 and 20. All histological procedures were the same.

That materials in the marking procedure are not the control stimuli for the reduction in MC numbers is evident. No significant differences in MC numbers existed between rats that had been marked with the permanent marker or those that had been marked with the water color. In addition, no significant differences in MC numbers existed between the marked animals and the nonmarked but stroked controls.

Although stimulation of the rat pup's rear dorsal surface appears to be critical for many social-emotional processes, from reflex defecation to

inter-rat behavior, the conclusion that this stimulation controlled the MC number variation is premature. First, why should the mean MC numbers of the handled and stroked rats (marked or nonmarked) in the split litter design fall between the mean MC numbers for the handled-stroked and nonhandled rats in the litter design? The only major differences between the stroked but nonmarked rats in the split litter design and the stroked rats in the litter design was the presence of marking smells in the former group. Do these smells attenuate the MC reduction effect?

Second, a comparison of two independent studies involving the same histological staining and counting procedures demonstrated a 50% reduction in MCs within rats that had been handled, injected, and weighed daily between postnatal ages 14 and 20, relative to rats that had been handled rarely. This implies that merely disruption of the nest or protracted restraint and handling of the pup (during stroking, shocking or injections) are the critical features. If indeed, as Schwartz (1975) and others suggest, brain histamine in the young brain originates primarily from mast cells, isolation of behavioral operations that alter these cells may be important.

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